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Increased β-Cyanoalanine Nitrilase Activity Improves Cyanide Tolerance and Assimilation in *Arabidopsis*

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ABSTRACT Plants naturally produce cyanide (CN) which is maintained at low levels in their cells by a process of rapid assimilation. However, high concentrations of environmental CN associated with activities such as industrial pollution are toxic to plants. There is thus an interest in increasing the CN detoxification capacity of plants as a potential route to phytoremediation. Here, *Arabidopsis* seedlings overexpressing the *Pseudomonas fluorescens* β-cyanoalanine nitrilase *pinA* were compared with wild-type and a β-cyanoalanine nitrilase knockout line (*ΔAtnit4*) for growth in the presence of exogenous CN. After incubation with CN, +*PfpinA* seedlings had increased root length, increased fresh weight, and decreased leaf bleaching compared with wild-type, indicating increased CN tolerance. The increased tolerance was achieved without an increase in β-cyanoalanine synthase activity, the other enzyme in the cyanide assimilation pathway, suggesting that nitrilase activity is the limiting factor for cyanide detoxification. Labeling experiments with [^{13}C] KCN demonstrated that the altered CN tolerance could be explained by differences in flux from CN to Asn caused by altered β-cyanoalanine nitrilase activity. Metabolite profiling after CN treatment provided new insight into downstream metabolism, revealing onward metabolism of Asn by the photorespiratory nitrogen cycle and accumulation of aromatic amino acids.

Key words: cyanide; β-cyanoalanine; cyanide detoxification; asparagine metabolism; nitrilase.

INTRODUCTION

Cyanide (CN) is a potent toxin because it binds the essential metal ion co-factors of metalloenzymes. CN is well known as an inhibitor of cytochrome C oxidase of the mitochondrial electron transport chain, but it also inhibits other enzymes, notably catalase, peroxidase, nitrate/nitrite reductase, superoxide dismutase, and Rubisco (Grossmann, 1996). Despite its toxicity, plants produce CN as part of their normal metabolism. Synthesis of the hormone ethylene from the precursor 1-aminocyclopropane-1-carboxylic acid (ACC) results in the stoichiometric production of CN (Peiser et al., 1984). To prevent self-poisoning, plants maintain an endogenous CN detoxification pathway. Early feeding experiments with ^{14}CN demonstrated that the radiolabeled carbon is incorporated first into β-cyanoalanine and then into Asn and to a much lesser extent Asp (Blumenthal-Goldschmidt et al., 1963; Lees et al., 1968; Ting and Zschoche, 1970). It is now established that a mitochondrial β-cyanoalanine synthase (CAS) catalyzes the addition of CN to cysteine to produce H_2S and β-cyanoalanine (Blumenthal et al., 1968; Hatzfeld et al., 2000; Watanabe et al., 2008), which is also a toxic metabolite in plants and animals (Pfeffer and Ressler, 1967; Howden et al., 2009a). β-cyanoalanine is then transported to the cytosol where its nitrile group is fully detoxified by hydrolysis to Asn or Asp by β-cyanoalanine nitrilase (Piotrowski et al., 2001; Piotrowski and Volmer, 2006). There are other potential routes of CN detoxification in plants but genetic and biochemical evidence indicates that flow through the β-cyanoalanine intermediate is the predominant pathway (Miller and Conn, 1980; Piotrowski and Volmer, 2006; García et al., 2010).

The endogenous plant cyanide detoxification system appears to have limited capacity having evolved principally to deal with the relatively small amounts of cyanide produced during metabolism. Upon exposure to elevated environmental CN (>50 μM), which can arise from both anthropogenic and natural sources (Alström and Burns, 1989; Ebbs et al., 2010; Blom et al., 2011), plants display growth inhibition,

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inhibition of transpiration, bleaching of leaves, and eventually death (Alström and Burns, 1989; Larsen et al., 2005; Blom et al., 2011). Nevertheless, plants are capable of assimilating exogenous CN (Ebbs, 2004) and experiments with wheat have shown that this assimilation rate increases under nitrogen limiting conditions (Ebbs et al., 2010). Consequently, phytoremediation has been proposed as a method of reducing environmental CN pollution. However, the effects of exposure to exogenous CN on plant metabolism have not been studied in detail and, to our knowledge, there has been no reported attempt to increase CN tolerance or assimilation rates through a genetic strategy.

Studies with *Arabidopsis* have shown that knockdown of *AtCysC1* (encoding the sole mitochondrial CAS) results in decreased CAS activity and accumulation of endogenously produced CN in the root but not the leaf (García et al., 2010). Increasing CN sources within the media also correlated with increased internal CN concentrations in the *Arabidopsis* CAS knockdown line (García et al., 2010). In contrast, no correlation was found between external and internal cyanide concentrations in wild-type *Arabidopsis* (García et al., 2010) or hydroponically grown rice seedlings (Yu et al., 2012). CAS activity has been shown to increase upon exposure to exogenous KCN in wheat (Machingura and Ebbs, 2010) and rice (Yu et al., 2012) and tobacco (Liang, 2003). These results suggest that, under conditions of moderate external CN exposure, endogenous CAS activity in plants is sufficient to avoid CN toxicity. However, the effect of CAS overexpression on plant CN tolerance remains to be investigated.

Previous work investigated the physiological function of the nitrilase gene *pinA* from *Pseudomonas fluorescens*, which was shown to have homology to the β-cyanoalanine nitrilase gene family from plants and a preference for β-cyanoalanine as a substrate (Howden et al., 2009a; Howden and Preston, 2009). Expression of *pinA* enabled *P. fluorescens* to grow in toxic concentrations of β-cyanoalanine and to use β-cyanoalanine as a sole nitrogen source. Furthermore, ectopic expression of *PfpinA* in *Arabidopsis* improved plant growth, particularly root growth, in the presence of exogenous β-cyanoalanine. In this study, the correlation between β-cyanoalanine nitrilase levels and CN tolerance was examined in *Arabidopsis* using T-DNA insertion knockdown (Δ*Atnit4*) and overexpression (+*PfpinA*) lines to establish the potential of transgenic manipulation of the plant cyanide detoxification pathway as a route to phytoremediation.

RESULTS

Increased Expression of β-Cyanoalanine Nitrilase Improves Cyanide Tolerance in *Arabidopsis*

Two *Arabidopsis* lines were previously established with altered expression of nitrilase genes encoding enzymes that metabolize β-cyanoalanine (Howden et al., 2009a). These lines showed altered tolerance to exogenous β-cyanoalanine. To assess whether these lines are also altered in their ability to grow in the presence of KCN, a robust assay was developed for plant seedlings that retains the volatile CN within growth media plates (see the 'Methods' section) and allows the physiological and metabolic effects of CN to be quantified. Seedlings were germinated and grown in vertical Murashige and Skoog (MS) media plates for 6 d then transferred to similar plates containing KCN and grown for an additional 5 d. To determine the relative toxicity of various CN concentrations, the increase in primary root length during incubation at 5 CN concentrations was compared between the mutant and wild-type lines (Figure 1A). We found that CN inhibits root elongation in wild-type *Arabidopsis* seedlings starting at concentrations as low as 10 μM. Increased CN concentrations correlated well with decreased root growth up to a concentration of 100 μM, at which point all root growth was inhibited.

At KCN concentrations above 0, the +*PfpinA* lines displayed significantly increased primary root growth compared with wild-type. In contrast, the Δ*nit4* line grew significantly more

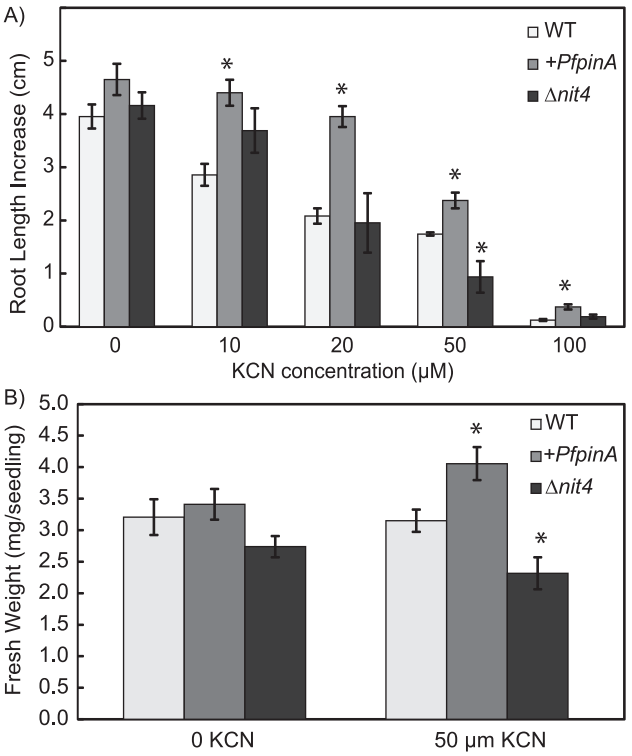

Figure 1. Growth of Wild-Type, +*PfpinA*, and *Δnit4 Arabidopsis* Lines after Incubation with KCN.

Six-day-old seedlings were incubated for 5 d in the presence of various concentrations of KCN.

(A) Tap root lengths were measured before and after the KCN incubation and the increase in root length is shown ($n = 6$).

(B) The averaged individual fresh weight of 10–20 seedlings after incubation with or without 50 μM KCN ($n = 9$). Error bars represent standard error. Asterisks indicate a significant difference from wild-type within that treatment ($p < 0.05$; (A) one-way ANOVA; (B) two-way ANOVA).